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Lysosomal enzyme binding to mouse P388D$_1$ macrophage membranes lacking the 215-kDa mannose 6-phosphate receptor: Evidence for the existence of a second mannose 6-phosphate receptor

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ABSTRACT **Mouse P388D$_1$ macrophages target newly synthesized acid hydrolases to lysosomes in spite of their lack of the 215-kDa mannose 6-phosphate (Man-6-*P*) receptor. We now report that these cells contain a membrane-associated Man-6-*P* receptor that is distinct from the previously described receptor. The new receptor binds lysosomal enzymes containing phosphomannosyl residues. This binding is inhibited by Man-6-*P* or by pretreatment of the lysosomal enzymes with alkaline phosphatase. Lysosomal enzyme binding occurs at neutral pH and dissociation of the bound ligand occurs at low pH values comparable to those found within endosomes or lysosomes. The new receptor differs from the 215-kDa Man-6-*P* receptor in two ways. It has an absolute requirement for divalent cations and is unable to bind *Dictyostelium discoideum* lysosomal enzymes, which contain methylphosphomannosyl residues rather than the usual phosphomannosyl monoesters. Based on the difference in cation requirement, we suggest that the 215-kDa receptor be referred to as Man-6-*P* receptor CI (cation independent) and the new receptor as Man-6-*P* receptor CD (cation dependent). We conclude that the Man-6-*P* receptor CD functions in the targeting of newly synthesized acid hydrolases to lysosomes in P388D$_1$ macrophages.**

Many cells sequester newly synthesized lysosomal enzymes into lysosomes by means of a recognition system involving phosphomannosyl residues (reviewed in ref. 1). Thus, the newly synthesized acid hydrolases acquire mannose 6-phosphate (Man-6-*P*) residues in the Golgi apparatus, and these residues serve as ligands for binding to a receptor (the Man-6-*P* receptor). The resulting complex is translocated to an acidic compartment (prelysosomal vesicles or lysosomes) where the low pH induces the release of the receptor-bound lysosomal enzymes. The lysosomal enzymes are then packaged into lysosomes and the receptor recycles back to the Golgi. The Man-6-*P* receptor has been purified from a number of sources and appears to be a single protein with an apparent molecular weight of 215,000 (2, 3). It binds lysosomal enzymes containing phosphomannosyl residues with high affinity, and this binding is independent of ions.

In previous studies we have identified and characterized a number of murine tissue culture cell lines that are deficient in the 215-kDa Man-6-*P* receptor (4). In spite of having no detectable or barely detectable receptor, the cells contained high levels of intracellular lysosomal enzymes that were localized to dense granules characteristic of lysosomes. The cells were shown to be incapable of taking up lysosomal enzymes from the media, excluding secretion–recapture as the mechanism for sequestering the lysosomal enzymes into lysosomes. Based on these data we suggested that the cells possess a pathway for the intracellular transport of acid hydrolases to lysosomes that is independent of the 215-kDa Man-6-*P* receptor. It was noted, however, that the lysosomal enzymes synthesized by these cells contained normal amounts of the phosphomannosyl recognition marker.

Since the sorting of lysosomal enzymes from secretory proteins in these cells seemed likely to be receptor-mediated, we sought conditions for demonstrating the binding of lysosomal enzymes to the membranes of these cells. As probes we prepared the lysosomal enzymes β-galactosidase and β-hexosaminidase from the secretions of murine P388D$_1$ cells, one of the receptor-negative lines. We now report that these two probes will bind to the membranes of cells lacking the 215-kDa Man-6-*P* receptor when divalent cations are present. This binding is saturable and behaves like a receptor-mediated process. Surprisingly, the binding is completely inhibited by Man-6-*P*. These results suggest that, in addition to the 215-kDa Man-6-*P* receptor, there is a second, ion-dependent Man-6-*P* receptor that participates in the delivery of acid hydrolases to lysosomes.

MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade. Sugar phosphates, D-mannose, 4-methylumbelliferyl-β-D-galactopyranoside, and 4-methylumbelliferyl-β-D-*N*-acetylglucosaminylpyranoside were from Sigma. *Hansenula holstii* (NRRL Y-2448) phosphomannan was a generous gift of M. Slodki (U.S.D.A. Northern Regional Laboratory, Peoria, IL). The pentamannosephosphomonoester was prepared as described in ref. 5. Homogenous *Escherichia coli* alkaline phosphatase was a gift of Milton Schlesinger (Washington University, St. Louis). Mannose–bovine serum albumin (mannose–BSA) containing 33–37 mol of sugar per mol of protein was kindly provided by Phil Stahl (Washington University, St. Louis).

Cells. Human I-cell fibroblasts were maintained as monolayers in α-minimal essential medium containing 10% fetal calf serum. When used for the preparation of lysosomal enzymes, mouse P388D$_1$ macrophages were cultured as monolayers in α-minimal medium containing 10 mM Hepes (pH 7), 2 mM sodium β-glycerophosphate, 2 mM sodium pyruvate, and 2 mM glutamine. Otherwise, they were grown in suspension in α-minimal medium containing 10% fetal calf serum. Cells were harvested at confluency or when maximal density was reached.

Isolation of Lysosomal Enzymes. β-Galactosidase and β-hexosaminidase were isolated from the culture medium of P388D$_1$ cells. The medium was first concentrated by using an Amicon YM-10 membrane, and the β-galactosidase was prepared as described (6). β-Hexosaminidase was prepared

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Abbreviations: Man-6-*P*, mannose 6-phosphate; TIU, trypsin inhibitor unit; BSA, bovine serum albumin.

as follows: after ammonium sulfate precipitation (75% saturation), the protein pellet obtained after centrifugation was dissolved in 50 mM sodium citrate, pH 4.8/150 mM NaCl/5 mM sodium β-glycerophosphate and dialyzed extensively against the same buffer. After clarification by centrifugation, the extract was applied to a 0.7 × 18 cm *p*-aminophenyl-β-D-*N*-acetylglucosamine-Sepharose4Bcolumn. β-Hexosaminidase was recovered by eluting with 50 mM Hepes, pH 7/0.3 M NaCl/5 mM sodium β-glycerophosphate. *Dictyostelium discoideum* β-hexosaminidase was prepared as described in ref. 4 and was a generous gift of A. Elhammer of this laboratory.

Membrane Preparation and Binding of Lysosomal Enzymes to Membranes. Cells (1–2 × 10^8) in suspension were collected by centrifugation and washed twice with 10 mM Tris·HCl, pH 7.4/150 mM NaCl. Monolayers were washed several times with the same buffer, and cells were harvested with a rubber policeman. Cells were resuspended in 5 ml of 50 mM Hepes, pH 7/150 mM NaCl/0.34 trypsin inhibitor unit (TIU)/ml of Trasylol and ruptured by sonication, using three 10-sec bursts (80 W) of a Bronwill sonicator. Lysates were centrifuged at 800 × *g* for 10 min and the postnuclear supernatant was adjusted to 0.5% saponin and 10 mM Man-6-*P*. After incubation on ice for 30 min, total membranes were pelleted by centrifugation at 100,000 × *g* for 30 min. The membrane pellets were then washed once with 5 ml of 50mH Hepes, pH 7/150 mM Nacl/0.5% saponin/0.17 TIU/ml of Trasylol/5 mM sodium β-glycerophosphate (membrane buffer) containing 10 mM Man-6-*P* and twice with 5 ml of the same buffer without Man-6-*P*. The final pellet was resuspended in 2 ml of membrane buffer. Under these conditions, the residual enzymatic activity in the membranes is <10% of the β-galactosidase or β-hexosaminidase activity bound in subsequent assays. Protein concentration was assayed with the Lowry method (7) after precipitation of an aliquot with trichloroacetic acid.

Binding of lysosomal enzymes was assayed as follows: usually 100 μg of membrane protein was incubated on ice for 90 min in the presence of lysosomal enzymes in 150 μl of 50 mM Hepes, pH 7/150 mM NaCl/0.5% saponin/0.17 TIU/ml of Trasylol/5 mM sodium β-glycerophosphate/10 mM $CaCl_2$/10 mM $MgCl_2$/10 mM $MnCl_2$ (incubation buffer). Membranes were then washed three times with 2.5 ml of the same buffer and collected by centrifugation at 100,000 × *g* for 30 min. The washed pellets were assayed for enzyme activity.

Enzyme Assays. β-Galactosidase and β-hexosaminidase were assayed for 30 min at 37°C in 0.5 ml of 0.1 M sodium citrate at pH 4 and pH 4.8, respectively, containing 0.2% Triton X-100 and either 1 mM 4-methylumbelliferyl β-galactoside or 1 mM 4-methylumbelliferyl *N*-acetyl-β-glucosaminide. The reaction was stopped by adding 0.5 ml of 0.5 M Na_2CO_3. One unit of β-galactosidase or β-hexosaminidase represents the release of 1 nmol of 4-methylumbelliferone in 1 hr.

Alkaline Phosphatase Treatment of β-Galactosidase. β-Galactosidase was incubated with 1 unit of *E. coli* alkaline phosphatase in the following buffer: 50 mM Hepes, pH 7/150 mM NaCl/10 mM $MgCl_2$/BSA at 1 mg/ml/leupeptin, antipain, chymostain, and pepstatin, each at 20 μg/ml/0.115 TIU/ml of Trasylol. Under these conditions alkaline phosphatase exhibits 80% of its activity measured at the usual pH of 8.

RESULTS

Binding of β-Galactosidase to P388D_1 Membranes. Membranes from P388D_1 cells were selected for use in the experiments designed to detect a second receptor involved in lysosomal enzyme targeting because these cells are totally devoid of the 215-kDa Man-6-*P* receptor (4, 8). Consequently, any lysosomal enzyme binding that occurs would have to be due to the presence of a receptor other than the 215-kDa Man-6-*P* receptor. We also assumed that the postulated receptor must be capable of recognizing the lysosomal enzymes synthesized by P388D_1 cells and therefore prepared β-galactosidase and β-hexosaminidase from the secretions of these cells. This was a convenient source of enzyme since the cells secrete about 70% of their newly synthesized enzymes (4). Finally, the binding experiments were carried out in the presence of the divalent cations $CaCl_2$, $MgCl_2$, and $MnCl_2$ since all of the carbohydrate-binding receptors with the exception of the 215-kDa Man-6-*P* receptor require calcium for ligand binding (9, 10).

Using these conditions the binding of β-galactosidase to P388D_1 membranes was readily detectable. Fig. 1 shows that the binding is dependent on the concentration of added ligand and membrane. The binding of β-galactosidase increased with the time of incubation, approaching a plateau at about 120 min when the assays were performed at 0°C (Fig. 1*C*). Surprisingly, the binding of β-galactosidase to the P388D_1 membranes was almost completely inhibited in the presence of 10 mM Man-6-*P*.

Inhibition of the Binding of β-Galactosidase. As shown in Table 1, Man-6-*P* is a relatively specific inhibitor of β-galactosidase binding. Fructose 6-phosphate gave partial inhibition at 1 mM, whereas the other sugar phosphates as well as mannose and mannose–BSA were without effect. Further, as shown in Fig. 2, Man-6-*P* is a very potent inhibitor of β-galactosidase binding, with 50% inhibition occurring at

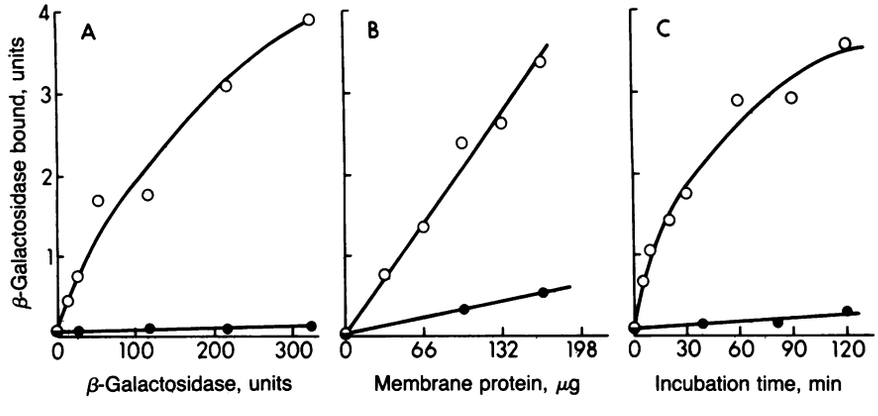

FIG. 1. Binding of β-galactosidase to P388D_1 macrophage membranes. Binding assays were carried out in the absence (○) or in the presence (●) of 10 mM Man-6-*P*. In *A* and *C*, 100 μg of membrane protein was utilized for each determination and in *B* and *C*, 250 units of β-galactosidase was added at each point.

Table 1. Inhibition of β-galactosidase binding to mouse $P388D_1$ membranes

Added inhibitor	Concentration	Binding of β-galactosidase	
		Units bound	% inhibition
None	—	4.29	—
Man-6-*P*	1 mM	0.14	97
Fructose 6-phosphate	1 mM	1.95	55
Glucosamine 6-phosphate	1 mM	5.09	—
Glucose 6-phosphate	1 mM	5.89	—
Mannose	10 mM	4.2	2
Mannose–BSA	50 μg/ml	3.9	9

Binding was measured by using 270 units of β-galactosidase in the absence or in the presence of the indicated inhibitors. After incubation on ice, the membranes were washed three times with the incubation buffer containing the same concentration of inhibitor.

10^{-5} M. In addition, pentamannose phosphomonoester derived by mild acid hydrolysis of *H. holstii* phosphomannan also inhibits β-galactosidase binding at low concentrations (Fig. 2). The lack of inhibition by mannose and mannose–BSA excludes the possibility that the ligand is binding to the mannose/fucose receptor known to be present in $P388D_1$ membranes (11).

Pretreatment of the β-galactosidase with *E. coli* alkaline phosphatase resulted in an 80% decrease in ligand binding to the $P388D_1$ membranes (Table 2). This result indicates that a phosphomonoester on the ligand is involved in binding to the membranes.

Effect of pH on Dissociation of Membrane-Bound β-Galactosidase. Fig. 3 shows the effect of pH on the release of β-galactosidase previously bound to $P388D_1$ membranes. As the pH is decreased below 5.5 there is a dramatic release of ligand, which is almost complete at pH 4.8. This type of pH-induced dissociation of ligand–receptor complexes is typical of receptors involved in the delivery of ligands to acidic compartments (12).

Divalent Cation Requirements. It has been reported that the binding of lysosomal enzymes to the Man-6-*P* receptor does not require the presence of cations (2, 13, 14). Since the

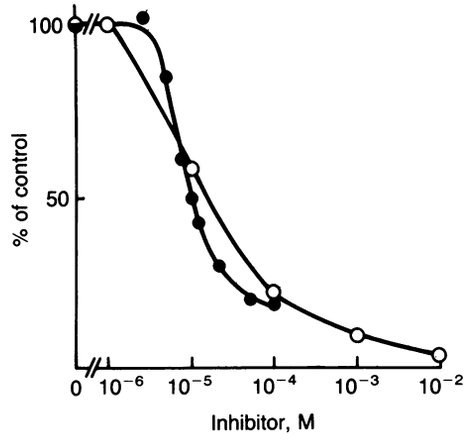

FIG. 2. Inhibition of β-galactosidase binding to $P388D_1$ membranes by Man-6-*P* or pentamannosephosphomonoester. $P388D_1$ membranes (100 μg of protein) were incubated either with 415 units of β-galactosidase and increasing concentration of Man-6-*P* (○) or with 210 units of β-galactosidase and various amounts of pentamannosephosphomonoester prepared from *H. holstii* phosphomannans (●). The control values for β-galactosidase binding were 3.8 units/0.1 mg of protein and 3.1 units/0.1 mg of protein, respectively.

Table 2. Effect of pretreatment of β-galactosidase with alkaline phosphatase on membrane binding

Preincubation	Units applied	Binding of β-galactosidase	
		Units bound	% inhibition
None	1087	6.7	—
β-Galactosidase + Buffer	1087	6.7	—
β-Galactosidase + Alkaline phosphatase	1087	1.3	82

The β-galactosidase was treated with or without *E. coli* alkaline phosphatase and then incubated with $P388D_1$ membranes (100 μg of protein) under standard assay conditions. The total amount of β-galactosidase bound to the membranes was then determined.

binding assays used in the current study were performed in the presence of calcium, magnesium, and manganese, we next investigated whether one or more of these divalent cations was absolutely necessary for ligand binding. As shown in Table 3, a very low level of β-galactosidase binding was observed when the binding assays were carried out without divalent cations or with 2 mM EDTA. The addition of calcium or magnesium ions resulted in a definite stimulation of binding, but optimal binding occurred in the presence of manganese ions. The effect of $MnCl_2$ concentration on β-galactosidase binding is shown in Fig. 4. Maximal stimulation of binding occurred between 7.5 and 10 mM. The addition of calcium and magnesium to assays containing 10 mM $MnCl_2$ did not result in a further stimulation of ligand binding (Table 3).

Binding of β-Hexosaminidase from Various Sources to $P388D_1$ Membranes. β-Hexosaminidase isolated from the secretions of $P388D_1$ cells also bound to the membranes of $P388D_1$ cells in a Man-6-*P*-inhibitable fashion, showing that more than one lysosomal enzyme is capable of binding to these membranes (Fig. 5*C*). This probe also bound to I-cell fibroblast membranes (Fig. 5*A*).

Previously we reported that β-hexosaminidase from the slime mold *D. discoideum* was unable to bind to $P388D_1$ membranes, whereas it bound readily to Man-6-*P* receptors on other cell types, including human fibroblasts (4). These

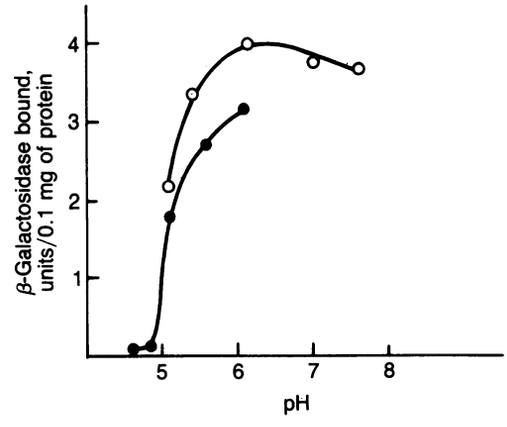

FIG. 3. Effect of the pH on the dissociation of membrane-bound β-galactosidase. β-Galactosidase (270 units) was first incubated on ice with 100 μg of $P388D_1$ membrane protein in 150 μl of incubation buffer at pH 7.0. After 90 min, the membranes were sedimented and washed once with 2.5 ml of incubation buffer at pH 7.0. The membranes were then reincubated on ice for 30 min in 2.5 ml of incubation buffer containing 50 mM sodium cacodylate (○) or 50 mM 4-morpholineethanesulfonic acid (●) adjusted to the indicated pH. After centrifugation, the membranes were washed once with the different buffers and assayed for β-galactosidase activity.

Table 3. Cation dependence of β-galactosidase binding to $P388D_1$ macrophage membranes

Condition	Binding of β-galactosidase	
	Units bound	%
No addition	1.37	17
2 mM EDTA	1.20	15
10 mM $CaCl_2$	4.42	53
10 mM $MgCl_2$	5.22	64
10 mM $MnCl_2$	8.22	100
10 mM (each) $CaCl_2$, $MgCl_2$, and $MnCl_2$	8.12	98

$P388D_1$ membranes (150 μg of protein) were incubated on ice for 90 min with 125 units of β-galactosidase in the absence or in the presence of EDTA, $CaCl_2$, $MgCl_2$, or $MnCl_2$ or in the presence of all three cations. Membranes were then washed three times with the incubation buffer containing the appropriate cation at a concentration of 10 mM and the amount of membrane-bound β-galactosidase was determined.

assays, however, were performed in the absence of added cations. In addition, we subsequently discovered that the Man-6-*P* residues on the *D. discoideum* β-hexosaminidase are in the form of methylphosphomannosyl diesters rather than Man-6-*P* monoesters (15). To determine whether the failure to detect binding to the $P388D_1$ membranes is due to the lack of cations and/or the inability of the receptor to recognize methylphosphomannosyl residues, the binding experiments were repeated in the presence of 10 mM $MnCl_2$. As shown in Fig. 5*B*, the *D. discoideum* β-hexosaminidase bound to the human fibroblast membranes and this binding was completely inhibited by Man-6-*P*. In contrast, there was no detectable binding of the probe to the $P388D_1$ membranes in the range of concentrations that give nearly maximal binding to the fibroblast membranes (Fig. 3*D*). At very high concentrations (100- to 200-fold higher than those required for binding to fibroblast membranes) some binding was observed, indicating that the Man-6-*P* receptor in the $P388D_1$ membranes recognizes *D. discoideum* β-hexosaminidase with a very poor affinity. Further evidence in support of this conclusion comes from the finding that the binding of β-galactosidase to $P388D_1$ membranes is completely inhibited

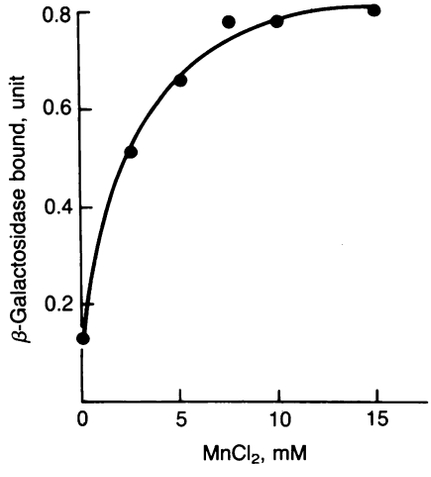

FIG. 4. Effect of $MnCl_2$ concentration on the binding of β-galactosidase to $P388D_1$ macrophage membranes. $P388D_1$ membranes (100 μg of protein) were incubated with 140 units of β-galactosidase and increasing concentrations of $MnCl_2$. After 90 min on ice, the membranes were washed three times with 2.5 ml of the incubation buffer containing the appropriate concentration of $MnCl_2$ and assayed for β-galactosidase activity.

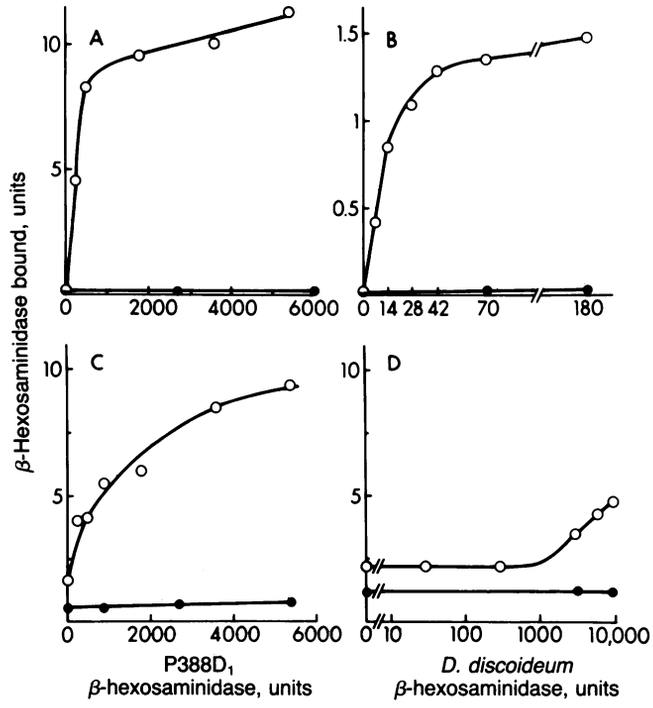

FIG. 5. Binding of β-hexosaminidase from different sources to human I-cell fibroblast and $P388D_1$ macrophage membranes. Binding assays were carried out by using human I-cell fibroblast membranes (*A* and *B*) or $P388D_1$ macrophage membranes (*C* and *D*) and β-hexosaminidase secreted by $P388D_1$ macrophages (*A* and *C*) or isolated from the differentiation medium of the slime mold *D. discoideum* (*B* and *D*) in the absence (○) or in the presence (●) of 10 mM Man-6-*P*.

by the addition of $P388D_1$ β-hexosaminidase but not by *D. discoideum* β-hexosaminidase (data not shown).

DISCUSSION

These data provide strong evidence for the existence of a Man-6-*P* receptor in $P388D_1$ membranes that is distinct from the well-characterized 215-kDa Man-6-*P* receptor. By using two lysosomal enzymes (β-galactosidase and β-hexosaminidase) isolated from the secretions of $P388D_1$ cells as probes, the membrane receptor was shown to have the following properties: (*i*) saturability; (*ii*) a requirement for the presence of phosphomonoester groups on the ligand, as demonstrated by inhibition of ligand binding after alkaline phosphatase treatment of the probe; (*iii*) specificity for Man-6-*P* and related compounds, such as pentamannosylphosphate, as determined by haptene inhibition studies; and (*iv*) sensitivity to pH, with stable binding of the ligand occurring at neutral pH followed by rapid dissociation at acidic pH values consistent with those found within endosomes and lysosomes (16). Each of these properties is also exhibited by the 215-kDa Man-6-*P* receptor (2, 13, 14, 17, 18). However, the two receptors differ in two major respects. First, the 215-kDa Man-6-*P* receptor does not require divalent cations for ligand binding, whereas the new receptor is dependent on ions, particularly $MnCl_2$. Second, the 215-kDa Man-6-*P* receptor binds *D. discoideum* lysosomal enzymes, whereas the new receptor recognizes these probes extremely poorly. This most likely reflects a subtle difference in the carbohydrate binding specificity of the two receptors, with the 215-kDa receptor being able to bind methylphosphomannosyl diesters in addition to Man-6-*P* monoesters, whereas the new receptor can only bind the phosphomonoesters. Further evidence for the existence of two distinct receptors

is the observation that P388D$_1$ cells do not synthesize any protein that can be immunoprecipitated by antisera to the 215-kDa Man-6-*P* receptor (8). Based on the difference in cation requirement, we suggest that the 215-kDa receptor be referred to as Man-6-*P* receptor CI (cation independent) and the new receptor as Man-6-*P* receptor CD (cation dependent).

The presence of a Man-6-*P* receptor in the P388D$_1$ cells provides an explanation for the targeting of acid hydrolases that occurs in these cells. It was noted in our previous study that although the P388D$_1$ cells secrete excessive quantities of lysosomal enzymes, they also synthesize large amounts of these enzymes so that the specific activity of the residual intracellular enzymes is actually higher than that found in some cells containing the 215-kDa Man-6-*P* receptor (4). In addition, the lysosomal enzymes were shown to contain the Man-6-*P* recognition marker, a finding that is verified in the present study. It seems likely, therefore, that the new Man-6-*P* receptor functions in P388D$_1$ cells to target newly synthesized acid hydrolases to lysosomes.

In preliminary experiments we have found that the new receptor is also present in other cell types, including those that contain the 215-kDa Man-6-*P* receptor. This raises the interesting question as to why cells would utilize two distinct Man-6-*P* receptors. The simplest explanation is that the two receptors serve to deliver newly synthesized acid hydrolases to different classes of lysosomes that participate in specialized cellular functions. Thus, both receptors would pick up newly synthesized acid hydrolases in the Golgi but deliver them to separate staging areas (acidified compartments) for packaging into the various types of lysosomes. If the level of the two receptors was regulated or if the receptors were able to distinguish between lysosomal enzymes (perhaps by recognizing different isomers of phosphorylated oligosaccharides), the cell would have the potential to control the flow of acid hydrolases to the different types of lysosomes.

It has been demonstrated previously that lysosomal organelles are heterogeneous (19–21). Of particular interest is the observation of Oliver that acinar cells contain a specialized set of lysosomes, the basal lysosomes, that occupy a distinct region in the cell and are acid phosphatase negative (21). In our previous study we noted that the lysosomal enzymes synthesized by P388D$_1$ cells retain their phosphomannosyl residues long after their arrival in the lysosomes, consistent with the enzymes being packaged into lysosomes that are deficient in acid phosphatase activity (4). This raises the possibility that the new Man-6-*P* receptor is involved in the targeting of acid hydrolases to lysosomes that are acid phosphatase negative, whereas the 215-kDa Man-6-*P* receptor sorts acid hydrolases to lysosomes which contain acid phosphatase.

Finally, it should be noted that there must be mechanisms for localizing acid hydrolases to lysosomes that are independent of the phosphomannosyl recognition marker. The evidence for this comes from studies of patients with I-cell disease (mucolipidosis II) and pseudo-Hurler polydystrophy (mucolipidosis III). The cells of these patients are characterized by a deficiency of UDP-GlcNAc:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase, resulting in an inability to synthesize the phosphomannosyl recognition marker (22–24). As a consequence, newly synthesized lysosomal enzymes are unable to bind to the Man-6-*P* receptors. In some cell types, such as fibroblasts, this results in secretion of the enzymes into the extracellular milieu rather than targeting to lysosomes (25). However, in other cells, such as hepatocytes, Kupffer cells, and leukocytes, there are nearly normal levels of lysosomal enzymes even though these cells are deficient in *N*-acetylglucosaminylphosphotransferase activity (26, 27). These cells, therefore, must be using a targeting mechanism distinct from the Man-6-*P* pathway.

The finding of a second Man-6-*P* receptor raises a number of fundamental questions concerning the sorting of lysosomal enzymes. As the next step toward understanding this system, it will be necessary to isolate and characterize this new receptor.

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